Acetyl Coenzyme A

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Principle

The best method for the determination of acetyl-CoA makes use of enzymatic acetylation of aromatic amines. This has the following advantages over other methods: the difference in free energy for the hydrolysis of the acylmercaptan and the carbon-amide bond is at least 4 kcal/mole and guarantees a quantitative conversion; with a suitable acceptor the reaction can be followed spectrophotometrically; the method for the purification of arylamine transacetylase*) is easily reproducible and requires little expenditure of time and material. Initially, sulphanilamide or *p*-aminobenzoic acid[1] were used as acceptor amines, then later, *p*-aminobenzene-sulphonic acid[2] and aminoazobenzene[3]: the most suitable is *p*-nitroaniline[4].

(1) $CH_3-CO\sim SCoA + H_2N-C_6H_4-NO_2 \xrightarrow[\text{transacetylase}]{\text{arylamine}} CH_3-CO-NH-C_6H_4-NO_2 + CoASH$

The λ_{max} for *p*-nitroaniline is 388 mμ and for the acetylated base λ_{max} is 318 mμ. The absorption of the nitroacetanilide is virtually zero at 405 mμ, while the molar extinction coefficient of *p*-nitroaniline at this wavelength is about 80% of the maximum value. Less than 0.01 μmoles acetyl-CoA/ml. (less than 8 μg./ml.) are easily detectable.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$, A. R.
3. Potassium hydrogen carbonate, $KHCO_3$, A. R.
4. Potassium hydroxide, A. R.
5. Perchloric acid, A. R., sp. gr. 1.67, *ca.* 70% (w/w)
6. Ethylene-diamine-tetra-acetic acid
   sodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (Titriplex III, Trilon B, Versene)
7. Thioglycollic acid, about 80% pure.
8. *p*-Nitroaniline, pure
   if necessary, recrystallize from water, m. p. 147.5° C.
9. Arylamine transacetylase
   Arylamine transacetylase is present in especially large amounts in pigeon liver[5]. If kept cold and dry an acetone-dried powder of this organ retains its activity and serves as the starting material for the purification of the enzyme (for further details, see Appendix, p. 424).

Preparation of Solutions

Prepare all solutions with metal-free, distilled water.

I. Potassium dihydrogen phosphate (0.2 M):
   Dissolve 2.722 g. KH_2PO_4 in distilled water and make up to 100 ml.

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*) Synonyms: arylamine acetyltransferase, arylamine acetokinase.

1) *F. Lipmann*, J. biol. Chemistry *160*, 173 [1945]. *N. O. Kaplan* and *F. Lipmann*, J. biol. Chemistry *174*, 37 [1948].
2) *S. P. Bessman*, quoted in[5].
3) *R. E. Handschumacher*, *G. C. Mueller* and *F. M. Strong*, J. biol. Chemistry *189*, 335 [1951].
4) *H. Tabor*, *A. H. Mehler* and *E. R. Stadtman*, J. biol. Chemistry *204*, 127 [1953].
5) *M. Bühler*, quoted in[6].

II. Phosphate buffer (0.2 M; pH 6.8):
Dissolve 1.361 g. KH_2PO_4 and 1.781 g. $Na_2HPO_4 \cdot 2H_2O$ in distilled water and make up to 100 ml.

III. Potassium hydroxide (*ca.* 8 N):
Dissolve 45 g. KOH in distilled water with cooling and make up to 100 ml.

IV. Potassium hydroxide (*ca.* 0.5 N):
Dilute 6.25 ml. 8 N KOH (solution III) to 100 ml. with CO_2-free water (boiled immediately before use)

V. Potassium hydrogen carbonate (*ca.* 1 M):
Dissolve 10 g. $KHCO_3$ in distilled water and make up to 100 ml.

VI. Perchloric acid (*ca.* 4 M):
Dilute 35 ml. 70% $HClO_4$ to 100 ml. with distilled water.

VII. Thioglycollic acid (*ca.* 0.1 M):
Dilute 0.085 ml. 80% thioglycollic acid to 10 ml. with distilled water.

VIII. Ethylene-diamine-tetra-acetate, EDTA (0.1 M):
Dissolve 1.86 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in distilled water and make up to 50 ml.

IX. *p*-Nitroaniline (0.002 M):
Dissolve 13.8 mg. *p*-nitroaniline in 1 ml. 96% ethanol and dilute to 50 ml. with distilled water.

X. Arylamine transacetylase:
Dissolve 5 to 10 mg. dry powder, according to the activity of the enzyme, in 0.2 ml. 0.2 M phosphate buffer, pH 6.8 (solution II). 25 units*), that is 0.01 to 0.02 ml., of this enzyme solution are required for each assay.

Stability of the solutions

Even in a deep-freeze the enzyme solutions have a limited stability. Therefore do not prepare more than a week's requirement of the enzyme solution. Solutions of acetyl-CoA are stable in the cold between pH 4 and 6. In alkaline solution a rapid hydrolysis occurs (refer to[6]) and even in strongly acid solution there is a gradual decrease of activity. Neutral solutions of thioglycollate are extremely rapidly autoxidized. The acid solution should be stored in a deep-freeze, but must not be used for longer than 10 to 15 days. The other solutions are stable for a long period if bacterial contamination is avoided (store in a refrigerator). The buffer and alkaline solutions should be stored in well stoppered polyethylene bottles.

Procedure

Extraction and deproteinization of the sample

To a solution of the sample in a centrifuge tube add a tenth of its volume of perchloric acid solution (VI) and mix well. After 3 min. neutralize most of the acid by dropping in 8 N KOH (solution III) (with shaking) and then adjust to between pH 6.3 and 6.7 by cautious addition of potassium hydrogen carbonate solution (V)**). Centrifuge off the precipitated protein

*) A unit[4]) is the amount of enzyme which changes the optical density at 420 mμ by 0.001 in 1 min. Assay conditions similar to those described under "Spectrophotometric measurements".

**) This avoids the addition of too much alkali to the solution, which would result in a partial hydrolysis of the acetyl-CoA. It is sufficient to check the pH with indicator paper, using a thin glass rod to remove a drop of the mixture. If the pH exceeds 7, then quickly add a few drops of potassium dihydrogen phosphate solution (I).

6) *K. Decker*: Die aktivierte Essigsäure. Ferd. Enke, Stuttgart 1959.

and potassium perchlorate at 3000 g for 5 min. Suck or pour off the supernatant as quantitatively as possible and transfer to a 5 ml. measuring cylinder. Wash the precipitate once with a little cold water, centrifuge and combine the washings with the supernatant. Use a portion of this solution without further treatment for the estimation.

Aqueous solutions containing acetyl-CoA can be stored for several days in the frozen state If acetyl-CoA is to be determined in tissues, then an especially thorough extraction must be carried out. It is usually necessary to concentrate the extract obtained. Refer to[7].

Spectrophotometric measurements

Wavelength: 405 mμ; light path: 1 cm.; final volume: 2 ml.; room temperature. Measure against the control.

Pipette successively into the cuvettes:

	Experimental	*Control*
sample	up to 1.10 ml.	—
phosphate buffer (solution II)	0.50 ml.	0.50 ml.
EDTA solution (VIII)	0.10 ml.	0.10 ml.
p-nitroaniline solution (IX)	0.10 ml.	0.05 ml.
thioglycollic acid solution (VII)	0.10 ml.	0.10 ml.
0.5 N KOH (solution IV)	0.02 ml.	0.02 ml.
distilled water	to 1.98 ml.	to 1.98 ml.

Mix well*) and read optical density E_1. Start the reaction by mixing into both cuvettes

0.02 ml. enzyme solution (X).

Follow the decrease in optical density until it is constant and then read the final optical density E_2.

Notes: The addition of *p*-nitroaniline to the control cuvette ensures that even if $E_2 = 0$, sufficient excess of acceptor is present in the experimental cuvette. Moreover, this allows the use of optimal *p*-nitroaniline concentrations, while at the same time working with the most sensitive part of the spectrophotometer scale. The sample should only contain sufficient acetyl-CoA (if necessary, determine by preliminary assay), so that ΔE is not larger than 0.300 to 0.400.

Calculations

According to p. 37, with an assay volume of 2 ml. and 1 cm. light path:

$$0.195 \times \Delta E \times \frac{V}{v} = \mu\text{moles acetyl-CoA in the whole sample}$$

where

V = total volume of the sample in ml.

v = portion taken for assay in ml.

$\Delta E = E_1 - E_2$

$\varepsilon = 1.025 \times 10^7 \text{cm.}^2/\text{mole}$, the molecular extinction coefficient of *p*-nitroaniline at 405 mμ.[8]

When the determination is started with 0.01 to 0.02 ml. enzyme solution it is unnecessary to correct for the dilution of the assay mixture.

*) With a small glass spoon bent at right angles, which is also used for addition of the enzyme.

7) *O. Wieland, G. Löffler, L. Weiss* and *I. Neufeldt*, Biochem. Z. *333*, 10 [1960].

8) *J. Knappe*, quoted in[6].

Example

An incubation mixture in which 0.15 μmoles acetoacetyl-CoA were cleaved to acetyl-CoA by 0.25 μmoles coenzyme A and β-ketoacylthiolase (volume 2.5 ml.) was quantitatively transferred to a centrifuge tube (final volume was 3.0 ml.). For deproteinization and neutralization to pH 6.6, 0.3 ml. perchloric acid solution (VI), 0.15 ml. KOH (solution III) and 0.04 ml. $KHCO_3$ solution (V) were required. After centrifuging, washing with 0.5 ml. water and re-centrifuging, the supernatants were combined. Total volume was 3.75 ml., of which 0.5 ml. was used for the determination.

$E_1 = 0.563$; $E_2 = 0.355$; $\Delta E = 0.208$.

$$0.195 \times 0.208 \times \frac{3.75}{0.5} = 0.304 \text{ μmoles acetyl-CoA in 3.75 ml. of the sample}$$

Other Determinations

By addition of the necessary enzymes and cofactors the same assay mixture can be used for the estimation of all substrates which can react to yield stoichiometric amounts of acetyl-CoA. These substrates include intermediates of fatty acid degradation and, after addition of Mg^{2+} and desmolase (β-hydroxy-β-methylglutaryl-CoA desmolase[9] or β-hydroxy-β-methylglutaryl-CoA cleavage enzyme[10]), β-hydroxy-β-methylglutaryl-CoA[7]:

(2) β-Hydroxy-β-methylglutaryl-CoA $\xrightarrow{\text{desmolase}}$ acetoacetate + acetyl-CoA

Sources of Error

The presence of some CoA derivatives (acetoacetyl-[11] and β-hydroxy-β-methylglutaryl-CoA[9]) can result in values which are too high, because the arylamine transacetylase preparation is not free from other enzymes. Acetoacetyl-CoA should be determined separately (see p. 425) and be subtracted from the value found with arylamine transacetylase (1 mole acetoacetyl-CoA gives 2 moles *p*-nitroacetanilide). The desmolase activity of the enzyme solution can be considerably reduced by repeated freezing and thawing. In the presence of EDTA the activity of this enzyme is minimal because of its requirement for magnesium. To determine acetyl-CoA in the presence of propionyl-CoA a method involving measurement of citrate synthesis must be used (refer to "Other methods of determination", p. 423).

In high concentrations free coenzyme A inhibits the acetylation reaction[4]: 0.1 μmole reduces the rate of the reaction by 50%. As the reaction involves the formation of free coenzyme A it is recommended not to take more than 0.05 μmoles acetyl-CoA for the determination.

Arylamine transacetylase is a SH-enzyme and it is completely inhibited by *p*-chloromercuribenzoate (10^{-5} M), but this inhibition is reversible[4]. A possible inhibition of the activity of the enzyme by traces of heavy metals must be reckoned with in the preparation of the assay mixture.

A high and unstable initial optical density can result from the presence of haemoproteins (absorption in the Soret Band). *Wieland*[7] reported that an interfering autoxidation of reduced flavins could be eliminated by gassing the assay mixture with CO_2 before the start of the reaction and by working with stoppered cuvettes.

Specificity

Arylamine transacetylase has a high specificity with regard to the acyl component. Butyryl-CoA is converted at only 4% of the rate measured with acetyl-CoA[4]. Palmityl-CoA is inactive and causes a 50% inhibition of the reaction with acetyl-CoA at a concentration of 10^{-5} M. Acetoacetyl-, β-hydroxy-β-methylglutaryl-, β-hydroxybutyryl- or crotonyl-CoA form no *p*-nitroacylanilides.

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9) *J. Knappe*, Diploma Thesis, Universität München, 1956.
10) *B. K. Bachhawat*, *W. G. Robinson* and *M. J. Coon*, J. biol. Chemistry *216*, 727 [1955].
11) *F. Lynen*, *K. Decker*, *O. Wieland* and *D. Reinwein* in *G. Popják* and *E. Le Breton*: Biochemical Problems of Lipids. Butterworths, London 1956, p. 142.

The enzyme is less specific with regard to the thiol component of the acetylmercaptan. The K_M value[12] for acetyl-CoA is 2.4×10^{-5} M and for *N.S*-diacetylcysteamine is 550×10^{-5} M. If acetyl-CoA has to be determined in the presence of the acetyl derivatives of pantetheine phosphate, pantetheine or *N*-acetylcysteamine, then the one of the following methods must be used.

Other Methods for the Determination of Acetyl-Coenzyme A

To avoid the disadvantages of the arylamine transacetylase reaction, especially the low substrate specificity (thiol component) and the lack of purity of the enzyme, as well as the slow reaction rate, acetyl-CoA can be determined in a combined spectrophotometric test according to equation (5).

(3) $\text{Acetyl-SCoA} + \text{oxalacetate}^{2-} + H_2O \rightleftharpoons \text{citrate}^{3-} + \text{CoASH} + H^+$

(4) $\text{L-Malate}^{2-} + \text{DPN}^+ \rightleftharpoons \text{oxalacetate}^{2-} + \text{DPNH} + H^+$

(5) $\text{L-Malate}^{2-} + \text{acetyl-SCoA} + \text{DPN}^+ + H_2O \rightleftharpoons \text{citrate}^{3-} + \text{DPNH} + 2H^+ + \text{CoASH}$

Reaction (4) is catalysed by malic dehydrogenase[13] and reaction (3) by the condensing enzyme of *Ochoa*[14, 15]. The preparation of the crystalline condensing enzyme from pig heart requires a well equipped enzyme laboratory and some experience. The enzyme is not yet available commercially. Because of this the determination of acetyl-CoA according to equation (5) is not given as a general method, but the principle of the assay is outlined below (for details, see[16]).

The equilibrium constant of the over-all reaction (equation 5) is $K' = 8.38 \times 10^3$ moles/l. at pH 7.2[15]. To ensure a quantitative conversion of acetyl-CoA a pH of 8.0 to 8.5 is chosen. A highly active enzyme must be used because of the danger of hydrolysis in this medium. The following assay mixture has proved best:

Up to 1.0 ml. neutralized acetyl-CoA solution
0.5 ml. 0.2 M tris buffer (pH 8.25)
0.1 ml. 0.2 M DL-malate solution (potassium salt)
0.05 ml. 0.01 M diphosphopyridine nucleotide (neutral sodium salt)
crystalline condensing enzyme (10 units according to *Ochoa*[16])
water to 1.99 ml.

Wavelength: 340 mμ or 366 mμ; light path: 1 cm.

Mix and read optical density E_1. Start reaction with 20 units[17] malic dehydrogenase (suspension in 2.6 M ammonium sulphate solution), mix and read optical density E_2.

$$0.322 \times \Delta E \times \frac{V}{v} = \mu\text{moles acetyl-CoA in the whole sample}$$

where

$\varepsilon = 6.22 \times 10^6$ cm.^{2}/mole, the molecular extinction coefficient[18] of DPNH at 340 mμ.
V = total volume of the acetyl-CoA sample in ml.
v = portion taken for the assay in ml.
$\Delta E = E_2 - E_1$

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12) *M. Grassl*, quoted in[6].
13) *F. B. Straub*, Hoppe-Seylers Z. physiol. Chem. *275*, 63 [1952].
14) *S. Ochoa, J. R. Stern* and *M. C. Schneider*, J. biol. Chemistry *193*, 691 [1951].
15) *J. R. Stern, S. Ochoa* and *F. Lynen*, J. biol. Chemistry *198*, 313 [1952].
16) *S. Ochoa* in *S. P. Colowick* and *N. O. Kaplan*: Methods in Enzymology. Academic Press, New York 1957, Vol. I, p. 687.
17) *S. Ochoa* in *S. P. Colowick* and *N. O. Kaplan*: Methods in Enzymology. Academic Press, New York 1957, Vol. I, p. 735.
18) *B. L. Horecker* and *A. Kornberg*, J. biol. Chemistry *175*, 385 [1948].

For measurements at 366 mμ $\varepsilon_{DPNH} = 3.3 \times 10^6 cm.^2/mole$ and therefore:

$$0.607 \times \Delta E \times \frac{V}{v} = \mu moles\ acetyl\text{-}CoA\ in\ the\ whole\ sample.$$

A third method for the enzymatic determination of acetyl-CoA is the arsenolysis in the presence of phosphotransacetylase from *Clostridium kluyveri*[19].

Non-enzymatic methods can also be used under certain conditions. For example, the determination as acetohydroxamic acid[6, 20], the nitroprusside reaction[21] and UV-spectroscopy[6, 19].

Appendix

Isolation of arylamine transacetylase[4]

Grind acetone powder of pigeon liver in a mortar with 10 parts by weight water. To 96 ml. of cold extract, add 76 ml. acetone, discard the precipitate and add 193 ml. acetone to the supernatant. Dissolve the precipitate in 15 ml. water, add 90 ml. Cγ-alumina gel (11 mg. dry weight/ml.) and centrifuge. Wash sediment with 100 ml. water and then elute the enzyme with 100 ml. 0.01 M potassium phosphate buffer (pH 7.8). Add 100 μmoles EDTA per 100 ml. and then lyophilize the solution. Specific activity: about 275 units/mg.

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19) *E. R. Stadtman* in *S. P. Colowick* and *N. O. Kaplan*: Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 935.

20) *F. Lipmann* and *L. C. Tuttle*, J. biol. Chemistry *159*, 21 [1945].

21) *F. Lynen*, Liebigs Ann. Chem. *574*, 33 [1951]; see also[6].